Proc. Natl. Acad. Sci. USA
Vol. 90, pp. 7686–7690, August 1993
Pharmacology

Amino acid residues essential for biological activity of a peptide derived from a major histocompatibility complex class I antigen

(glucose transport/ordered structure)

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Contributed by Avram Goldstein, May 12, 1993

ABSTRACT **The stimulatory activity of peptides from the α1 domain of the major histocompatibility complex (MHC) class I antigen on adipose cell glucose transport was previously shown to require a preformed, ordered conformation of the peptide. The two peptides studied previously were D^k-(61–85) (ERETQIAKGNEQSFRVDLRTLLRYY) and D^k-(69–85). We now show that systematic alanine substitution in D^k-(69–85) identifies residues that are essential for biological activity. Ordered structure of the peptides, estimated by circular dichroism, was found in all peptides with activity, but with a complex variety of spectra. Inactive peptides were in either a random coil or an ordered structure. Ordered structure, therefore, is not sufficient for activity. The peptides self-interact in the absence of cells and form aggregates that precipitate upon centrifugation. The tendency to aggregate is correlated with biological potency. Only MHC class I molecules have significant homology to the peptides studied here. The peptide self-interaction suggests that the biological effects in cells, which result from inhibition of receptor and transporter internalization, may be due to the binding (tantamount to self-interaction) of the peptide to the homologous sequences in the α1 domain of the MHC class I molecule.**

Previous studies demonstrated that certain peptides derived from the α1 domain of major histocompatibility complex (MHC) class I antigens [e.g., D^k-(61–85)] enhance insulin-stimulated glucose transport in adipose cells. This effect is due to an increased number of active insulin receptors (1) and glucose transporters (13) on cell surfaces consequent to inhibition of receptor and transporter internalization (1–3). D^k-(61–85) is active only if it has assumed an ordered conformation prior to its interaction with cells (2). The initial findings were with 25-residue peptides, but it was found subsequently that the eight N-terminal residues could be removed without loss of activity (1). We now identify residues in D^k-(69–85) that are essential for activity and others that are not. In addition, we show that while inactive peptides may adopt either ordered structure or random coil conformation, active peptides must have an ordered structure. Peptide molecules in ordered structure interact with themselves to a greater or lesser extent, forming aggregates that precipitate upon centrifugation. Biological activity of the peptides is correlated with the tendency to form aggregates. We discuss the significance of the observations in light of the fact that the peptides have sequence similarity only to MHC class I molecules (4, 5) among known protein sequences.

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MATERIALS AND METHODS

Glucose Transport in Adipose Cells. The biological activity of the peptides was measured by their effect on glucose uptake in rat adipose cells as described elsewhere (1). Briefly, rat adipose cells were obtained from epididymal fat pads and suspended in Krebs–Ringer Hepes buffer with 5% bovine serum albumin at a lipocrit of 10% (final). The peptide effect was measured in cells maximally stimulated with insulin (8 nM). After equilibration at 37°C for 30 min the cells were incubated for 30 min at 37°C with buffer (basal), 8 nM insulin, or 8 nM insulin plus peptide. D-[^{14}C]Glucose was added, and the cells were incubated for an additional 30 min and harvested on oil. Biological activity was measured by a dose–response curve to interpolate the EC_{50} value in the usual way, taking the maximum enhancement of insulin effect (about 40% over the insulin-only maximum) as 100%. Most of the peptides were not tested at concentrations higher than 30 μM. Peptides that enhanced the maximum insulin effect by less than 20% at 30 μM were considered inactive. Accordingly, three categories of peptides were defined: those with full activity, $EC_{50} < 10$ μM; reduced activity, 10 μM $\leq EC_{50} \leq 30$ μM; no activity, $EC_{50} > 30$ μM.

Peptides. The peptides were assembled stepwise either on a phenylacetamidomethyl (PAM) resin using the *t*-Boc NMP/HOBt protocol of an Applied Biosystems model 430A peptide synthesizer or on a *p*-alkoxybenzyl alcohol (Wang) resin using a modified Fmoc/BOP protocol of a Milligen/Biosearch Model 9050 synthesizer. The side-chain-protecting groups were as follows: *t*-Boc chemistry, N^g-mesitylene-2-sulfonyl for Arg, β-cyclohexyl ester for Asp, γ-benzyl ester for Glu, N^ϵ-2-chlorobenzyloxycarbonyl for Lys, *O*-benzyl for Ser and Thr; and *O*-2-bromobenzyloxycarbonyl for Tyr; Fmoc chemistry, 4-methoxy-2,3,6-trimethylbenzenesulfonyl for Arg, β-*t*-butyl ester for Asp, γ-*t*-butyl ester for Glu, N^ϵ-*t*-butoxycarbonyl for Lys, and *O*-*t*-butyl for Ser, Thr, and Tyr. The *t*-Boc-assembled peptides were deprotected/cleaved from the solid support by using HF in the presence of anisole, ethanedithiol, and dimethyl sulfide as scavengers. After conversion of the hydrofluoride to the acetate salt by ion-exchange column chromatography, the peptides were purified to greater than 98% homogeneity by preparative high-performance liquid chromatography using a Vydac C_{18} (2.2 × 25 cm) column and appropriate linear gradients of 0.1% trifluoroacetic acid (TFA)-buffered acetonitrile in 0.1% aqueous TFA. The Fmoc-assembled peptides were deprotected/cleaved from the resin by using TFA in the presence of thioanisole, ethanedithiol, water, and phenol as scavengers and were purified by preparative high-performance liquid chromatography as described above. The desired peptides

Abbreviation: MHC, major histocompatibility complex.

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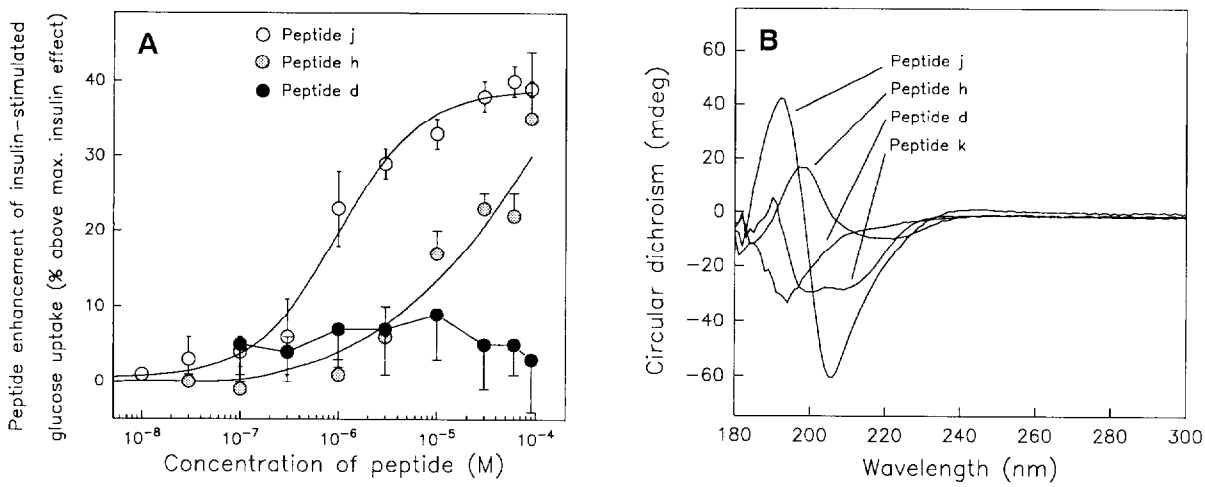

FIG. 1. (*A*) Representative dose–response curves for peptides with full biological activity (○), reduced activity (◎), or no activity (●). The values are mean ± SEM of three experiments with triplicate samples at each point. (*B*) CD spectra for 1 mM solutions of the three peptides in *A* and of peptide k. The measurements for each peptide were done twice at room temperature. The results of one set of experiments are shown.

were confirmed by sequence analysis, amino acid composition, and fast atom bombardment mass spectrometry. We reported previously that the MHC-derived peptides may occur in both an active and an inactive conformation. Accordingly, the peptides used in the present study were activated by incubation of 1 mM stock solution at 37°C in 0.1 M NaCl overnight, as described (2).

Circular Dichroism (CD). CD spectra were recorded on a Jasco (Easton, MD) J-600 calibrated against *d*-camphorsulfonic acid using $\Delta\varepsilon$ (290.5 nm) = +2.38 $M^{-1}\cdot cm^{-1}$. Rectangular cuvettes with path lengths of 0.01 cm were used for recording spectra of 1 mM peptide stock solutions.

Aggregation. Peptide stock solution was diluted in Krebs–Ringer Hepes pH 7.2 buffer to 30 μM, incubated (30 min, 37°C), then centrifuged at 12,000 × *g* for 10 min. The amount of peptide remaining in solution was measured spectrophotometrically by absorbance at 278 nm (ε = 1200 $M^{-1}\cdot cm^{-1}$ per tyrosine residue).

RESULTS

Biological Activity. We analyzed D^k-(62–85) and D^k-(69–85) by systematic replacement of residues with alanine (alanine scan) (6–8) to assess the importance of each residue for

Table 1. Sequence, biological activity, and aggregation of MHC class I-derived peptides

Peptide	Code	Sequence	Residue(s) replaced	EC_{50},* μM	Aggregation,† %
		62 65 70 75 80 85			
D^k-(62–85)	a	RETQIAKGNEQSFRVDLRTLLRYY		2	70 ± 2
D^k-(69–85)	b	GNEQSFRVDLRTLLRYY		5	NT
Single substitutions					
[Ala^{71}]D^k-(69–85)	c	GNAQSFRVDLRTLLRYY	E	1	65 ± 6
[Ala^{74}]D^k-(62–85)	d	RETQIAKGNEQSARVDLRTLLRYY	F	>30	33 ± 4
[Ala^{78}]D^k-(69–85)	e	GNEQSFRVDARTLLRYY	L	30	39 ± 6
[Ala^{81}]D^k-(69–85)	f	GNEQSFRVDLRTALRYY	L	>30	64 ± 6
[Ala^{82}]D^k-(69–85)	g	GNEQSFRVDLRTLARYY	L	>30	35 ± 4
[Ala^{83}]D^k-(69–85)	h	GNEQSFRVDLRTLLAYY	R	30	91 ± 1
[Ala^{84}]D^k-(69–85)	i	GNEQSFRVDLRTLLRAY	Y	10	89 ± 2
[Ala^{85}]D^k-(69–85)	j	GNEQSFRVDLRTLLRYA	Y	1	88 ± 5
Double substitutions					
[$Ala^{68,75}$]D^k-(62–85)	k	RETQIAAGNEQSFAVDLRTLLRYY	K, R	>30	44 ± 6
[$Ala^{69,76}$]D^k-(62–85)	l	RETQIAKANEQSFRADLRTLLRYY	G, V	30	42 ± 4
[$Ala^{70,77}$]D^k-(62–85)	m	RETQIAKGAEQSFRVALRTLLRYY	N, D	7	72 ± 4
[$Ala^{72,79}$]D^k-(62–85)	n	RETQIAKGNEASFRVDLATLLRYY	Q, R	>30	54 ± 4
[$Ala^{73,80}$]D^k-(62–85)	o	RETQIAKGNEQAFRVDLRALLRYY	S, T	>30	59 ± 4
Other peptides					
HLA-A2-(69–85)	p	AHSQTHRVDLGTLRGYY		>30	NT
HLA-A2-(69–76)-D^k-(77–85)	q	AHSQTHRVDLRTLLRYY		>30	NT
D^k-(69–76)-HLA-A2-(77–85)	r	GNEQSFRVDLGTLRGYY		10	NT
HLA-B27-(69–85)	s	AKAQTDREDLRTLLRYY		>30	NT
[Phe^{74}]HLA-B27-(69–85)	t	AKAQTFREDLRTLLRYY		1	NT

*EC_{50} value for glucose uptake as measured in the rat adipose cell assay. Maximal peptide effect in cells fully stimulated by insulin was 40% enhancement over insulin alone. Peptides giving less than 20% enhancement at 30 μM were considered inactive (EC_{50} > 30 μM).

†Aggregation was measured by centrifugation of 30 μM peptide solution in Krebs–Ringer Hepes for 10 min at 12,000 × *g* and the amount of peptide remaining in solution was determined spectrophotometrically. The numbers indicated are percent precipitated and are mean ± SEM of three experiments. NT, not tested.

biological activity. Fig. 1*A* shows three typical dose–response curves for stimulation of glucose uptake, for a fully active peptide (j), a peptide with reduced activity (h), and an inactive peptide (d). (See Table 1 for alphabetical coding.) Table 1 presents the potency of D^k-(62–85) and D^k-(69–85), of 13 peptides in the alanine scan, and of 5 additional peptides of interest. Substitution for Phe^{74} (d), Leu^{81} (f), and Leu^{82} (g) each resulted in loss of activity. Peptide d was completely inactive even at 90 μM, the highest concentration that was tested. Peptides with alanine instead of Leu^{78} (e), Arg^{83} (h), or Tyr^{84} (i) all had reduced activity compared with D^k-(69–85) (b). Replacement of Glu^{71} (c) or Tyr^{85} (j) yielded peptides that were even more potent than the original; this is an interesting result in particular for peptide j, as we had shown previously that C-terminal truncation by deleting Tyr^{85} results in considerable loss of activity (2).

The alanine scan with double residue changes showed (in m) that neither Asn^{70} nor Asp^{77} is important for activity. Peptide l had reduced activity, but the data do not allow a conclusion as to whether Gly^{69} or Val^{76} is most important. The three other peptides with double alanine substitutions (k, n, o) were all inactive. In peptide k the essential residue is likely to have been Arg^{75}, as residues 62–68 can be deleted entirely (cf. a and b) without loss of activity. The data did not allow a decision as to whether the inactivity of peptides n and o was due to substitution of only one or both of the residues. However, as the chimeric peptide r, with Gly^{79}, was moderately active, Gln^{72} seems more responsible than Arg^{79} for the loss of activity in peptide n.

The essential role of Phe^{74} is shown not only by the inactivity of d, and of both p and q (which contain many of the other residues shown to be essential), but most dramatically by the fact that the inactive human peptide s became fully active when Asp^{74} was changed to Phe^{74} (peptide t).

CD. We showed previously (2) that D^k-(61–85) is active only if it has assumed an ordered conformation prior to interaction with cells. Therefore residues identified in the alanine scan as important for biological activity might be essential for maintaining an ordered structure, for interaction with a binding site, or for both. Measurements at 1 mM in 0.1 M NaCl yielded a variety of complex CD spectra, so that simple classification into recognized structures [such as α-helix (9, 10)] was often not possible.

Fig. 1*B* shows typical CD spectra of four peptides (three of them the same as in Fig. 1*A*), selected to show the variable features. Peptide j, which is fully active, has a spectrum with maxima at 205 nm (negative) and 195 nm (positive), suggesting a high content of ordered structure. The original unsubstituted peptides a and b (see ref. 2) as well as the two other most active peptides (c and t) have a similar positive CD signal at 195 nm.

Peptides h and k, with reduced activity and no activity, respectively, have CD spectra with both a positive and negative maximum, but without the typical profile of peptide j. Peptides e, i, and r, all of which have reduced activity, and the inactive peptides (f, n, o, and s) also fall into this category. The CD spectrum of peptide m is also in this category, although its activity is comparable to that of peptide b. Peptides g and q (no activity) and peptide l (reduced activity) have spectra with a negative maximum, no positive maximum, but with an indication of some molecules with ordered structure.

Only the inactive peptides d and p have the typical spectrum of a random coil, with a negative maximum at 195 nm.

Analysis of the data in Fig. 2*A* shows a correlation between the degree of ordered structure as estimated by CD and the biological activity ($\chi^2 = 10.6$; $P < 0.05$), by a conservative test ignoring the rank order of categories in the 3 × 3 contingency table.

Aggregation. Table 1 shows the extent of aggregation of the various peptides. The scatter diagram in Fig. 2*B* indicates a positive correlation ($r = 0.56$; $P < 0.05$) of biological activity with the ability of the peptides to self-interact and form aggregates. Fig. 2*C* shows that peptide self-interaction (aggregation) and the degree of ordered structure also are correlated ($r = 0.49$; $P < 0.05$).

DISCUSSION

The present results, summarized in Fig. 3*A*, are consistent with and extend our earlier observations (2) on the gain and loss of activity associated with a reversible conformation change of D^k-(61–85), which is active only if it assumes an ordered structure prior to its interaction with cells. In the present series, ordered structure was correlated with potency, and all peptides with activity had ordered structure.

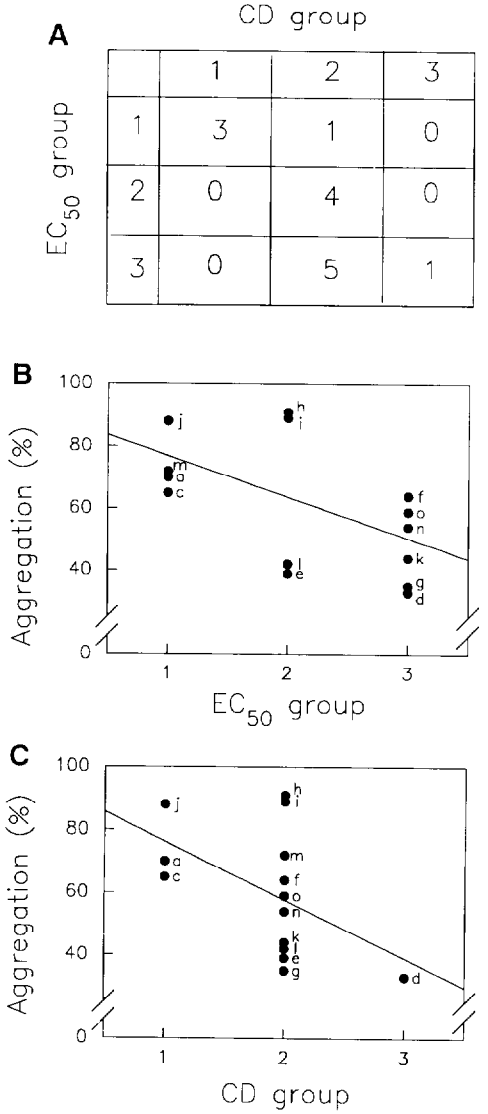

FIG. 2. Correlation between biological activity, ordered structure, and aggregation. (*A*) Biological activity and CD signal. Peptides were categorized into three groups according to EC_{50} values (see also *Materials and Methods*): 1, full activity; 2, reduced activity; 3, no activity. For CD spectra, three groups were defined: 1, spectrum comparable to that of peptide j (see Fig. 1*B*); 2, intermediate spectrum; 3, random coil. (*B*) Biological activity and aggregation. (*C*) CD spectra and aggregation. The three groups for biological activity and CD spectra are the same as in *A*. Aggregation values are from Table 1. Regression lines are shown.

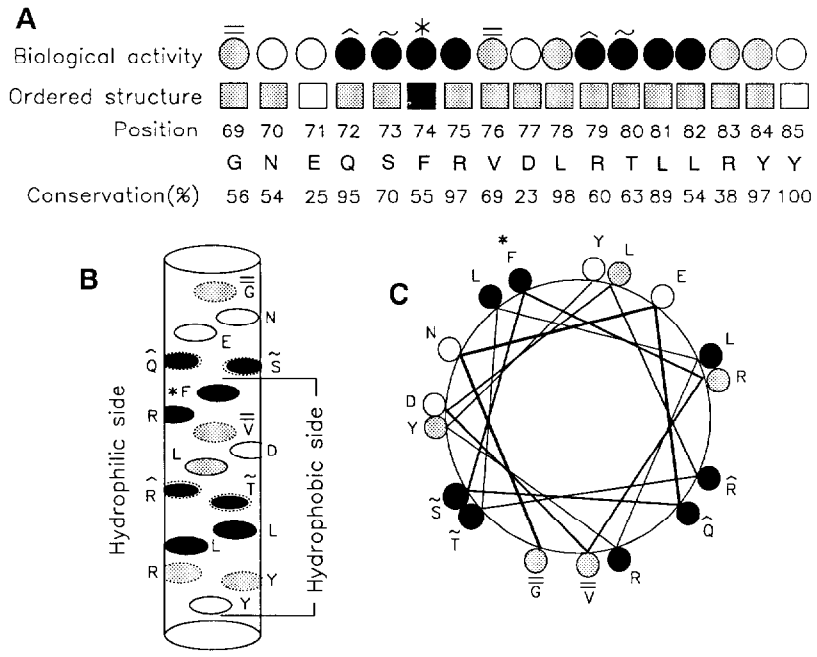

FIG. 3. Summary of importance of each residue in D^k-(69–85) for biological activity and ordered structure in the MHC class I peptides. (*A*) Residue positions at which alanine substitution has no effect on activity (○) or ordered structure (□); reduced activity (◎) or reduced CD signal for ordered structure (▣); and complete loss of activity (●) or ordered structure (■). ∧, ~, and = each indicate pairs of residues that result in reduced or complete loss of activity, it being unclear which residue is critical in each pair. * indicates residue position at which alanine substitution results in complete loss of both ordered structure and activity. Degree of phylogenetic conservation of each residue in the MHC class I molecule was estimated from 95 sequences in eight species. (*B*) Helical rod, assuming an α-helix for the ordered structure. Symbols for biological activity are as in *A*. Residues encircled by a solid line are in the front of the rod; those encircled by a dotted line are on the back side. Hydrophobic and hydrophilic surfaces of the peptide are indicated; the hydrophobic surface is more to the front than to the side. (*C*) Helical wheel with symbols for biological activity as in *A*.

However, not all peptides with ordered structure were active (cf. peptide k). Ordered structure is thus necessary but not sufficient for biological activity. Phe^{74} seems absolutely essential for the ordered structure. This is especially striking, not only because its replacement by alanine results in loss of both ordered structure and activity in the mouse peptide but also because introduction of Phe^{74} in a human sequence (in peptide t) restores ordered structure and fully activates an otherwise inactive peptide (s).

The systematic alanine substitutions show that replacement of residues required for full activity does not necessarily affect the ordered structure. This suggests that—given an ordered structure—the residues required for activity are part of the peptide domain that interacts with a binding site on the cell membrane. The helical rod and wheel depictions of D^k-(69–85) (peptide b), based on the assumption of an α-helix (Fig. 3 *B* and *C*), reveal an amphipathic structure. Interestingly, residues essential for activity (given an α-helical conformation) are primarily located on the hydrophilic side. Phe^{74}, which is essential for ordered structure, is situated on the hydrophobic side. All conclusions drawn from the alanine scan assume that the function of each residue is independent of the other residues in the peptide.

The significant correlation of potency with the tendency to aggregate suggests, on its face, that aggregates are the biologically active form. However, a more attractive explanation is that a monomer, dimer, or oligomer is the active form and that the property of a peptide that is responsible for self-interaction (aggregation) is also responsible for biological activity. If this were true, it would follow that true EC_{50} values based on unaggregated peptide concentrations are smaller than those given in Table 1, which are based on total peptide concentration—i.e., the true potency is greater, the greater the fraction aggregated.

Search of the GenBank, EMBL, CASBIO, and PIR protein sequence data bases on April 12, 1989 indicated that only MHC class I molecules have sequence similarity greater than 70% to the D^k-(61–85) peptide, and the greatest similarity of a protein other than MHC class I is only 32% (collagen α1 chain precursor). It is interesting, however, that the degree of phylogenetic conservation of the residues in the D^k-(69–85) domain of MHC class I is unrelated to the critical importance of those residues for biological activity in our studies (Fig. 3*A*). Conservation of residues in various MHC class I molecules may reflect a critical role in maintaining three-dimensional structure of the protein, whereas activity in our studies depends on interaction with a cell component that mediates inhibition of receptor internalization (2, 13).

We suggest two hypotheses:

(*i*) The peptides inhibit receptor internalization by competing with the α1-helix of MHC class I for a binding site on the cell surface and thus block a putative normal action of MHC class I in promoting receptor internalization.

(*ii*) The peptides bind to the α1-helix of MHC class I and thus sterically block its putative normal action, as above. The correlation of potency with tendency to self-interact may favor this hypothesis because a part of the α1-helix is identical to the D^k-(61–85) peptide. Thus, binding to this domain of MHC class I protein would be tantamount to self-interaction. Moreover, it is known that MHC class I molecules themselves may interact and form dimers, tetramers, and perhaps even larger oligomers in the membrane (11, 12).

Both hypotheses imply that MHC class I molecules, which function in the immune system to transport antigenic peptides to the cell surface, may also play a role in receptor recycling.

The authors thank Dr. Walter A. Baase (University of Oregon, Eugene), Dr. Barbara Dengler (University of California, Berkeley), and Dr. Marcos F. Maestre (Lawrence Berkeley Laboratory) for valuable help and advice for the CD studies; Prof. Morten Simonsen (University of Copenhagen) for valuable suggestions and comments; Tracy Cosgrove, Karen Frisella, and Alan Gubler for their expert

technical assistance; and Joyce Lilly (Johns Hopkins University, Baltimore) for synthesis of several peptides.

1. Stagsted, J., Reaven, G. M., Hansen, T., Goldstein, A. & Olsson, L. (1990) *Cell* **62,** 297–307.
2. Stagsted, J., Baase, W. A., Goldstein, A. & Olsson, L. (1991) *J. Biol. Chem.* **266,** 12844–12847.
3. Stagsted, J., Ziebe, S., Satoh, S., Holman, G. D., Cushman, S. W. & Olsson, L. (1993) *J. Biol. Chem.* **268,** 1770–1774.
4. Bjorkman, P. J., Saper, M. A., Samraoui, B., Bennett, W. S., Strominger, J. L. & Wiley, D. C. (1987) *Nature (London)* **329,** 506–512.
5. Madden, D. R., Gorga, J. C., Strominger, J. L. & Wiley, D. C. (1991) *Nature (London)* **353,** 321–325.
6. Cunningham, B. C. & Wells, J. A. (1989) *Science* **244,** 1081–1085.
7. Cunningham, B. C. & Wells, J. A. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 3407–3411.
8. Wells, J. A. (1991) *Methods Enzymol.* **202,** 390–411.
9. Woody, R. W. (1985) in *The Peptides*, ed. Hruby, V. J. (Academic, New York), Vol. 7, pp. 15–114.
10. Johnson, W. C. (1990) *Proteins* **7,** 205–214.
11. Chakrabarti, A., Matko, J., Barisas, B. G. & Edidin, M. (1992) *Biochemistry* **31,** 7182–7189.
12. Krishna, S., Benaroch, P. & Pillai, S. (1992) *Nature (London)* **357,** 164–167.
13. Stagsted, J., Olsson, L., Holman, G. D., Cushman, S. W. & Satoh, S. (1993) *J. Biol. Chem.* **268,** in press.